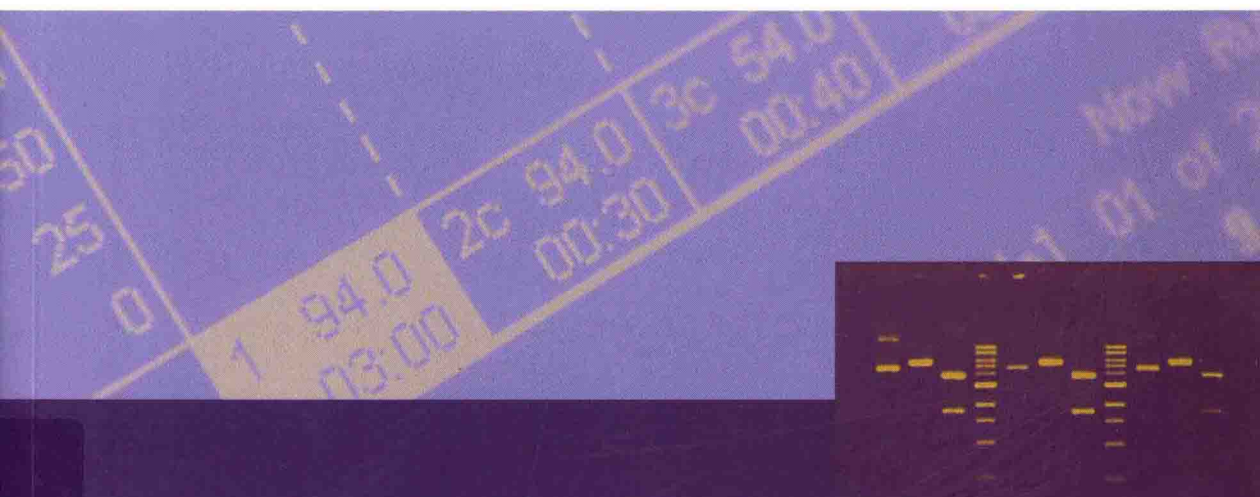
Essential Experiments for Molecular Biology

A Student's Guide

Shuping ZHANG
Peng LI



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Shuping ZHANG
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CENTER FOR LIFE SCIENCES

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The cover art on the back cover was drawn with a three-color palette of bacterial colonies expressing fluorescent proteins derived from GFP and dsRed.The artwork was created by Yuning Zhang, a student of the School of Life Sciences, Tsinghua University, as a class activity for a molecular biology laboratory course.

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Preface

Molecular biology is one of the most important disciplines in the life sciences. Since 1953, a revolution led by molecular biology has taken place and has resulted in extensive implications in many fields of the biological sciences. A major impetus behind this revolution is the development of powerful molecular biology techniques that have led to innumerable exciting breakthroughs in understanding biological activities and mechanisms. Currently, molecular biology techniques are being widely used in all disciplines in the life sciences.

This guidebook, *Essential Experiments for Molecular Biology: A Student's Guide*, is designed to expose undergraduates to a set of standard, basic molecular biology research techniques, including DNA extraction, electrophoresis, enzyme digestion, PCR, DNA recombination, RNA manipulation, and molecular hybridization. Although each can stand alone as an independent experiment, most of the experiments are interrelated because the main body of this coursework is derived from an actual research project. This type of course design allows students to obtain a systemic concept of conducting biological research so that they can thereafter easily adapt what they learn to real research projects. After finishing this course, students of various majors will understand the fundamental principles of molecular biology techniques and be able to use these methods or combine them with other skills to design an integrated molecular biological experiment. This book is suitable for use in a one-semester course for undergraduates (experiments 9-12 are optional) and as a reference book for postgraduates and researchers.

The preparation of the textbook is the result of collaborations with many coworkers and teaching assistants over the years. The laboratory assistant Yingzi Li, Wen Peng and teaching assistants Ying Tang, Qiang Zhou, Lu Zhou, Jian Yan, Ruiyan Wang and Suping Zhang were instrumental in collecting and preparing materials during the initiation of this book. We thank Prof. Jinyuan Liu and Dr. Hongxiu Ning for the help during the initiation of the course and thank our students who provided critical evaluations and suggestions. We are grateful for support from the Tsinghua University-Peking University Joint Center for Life Sciences. We are especially indebted to the guidance provided by Li Wang, Xinjing Gao and Higher Education Press. Special thanks go to Dr. Kary Mullis for permission to use his photo. We would also like to express our appreciation to all of the companies whose materials or information are

used in this textbook (Agilent Technologies Genomics, EMD Millipore, Life Technology, TIANGEN, TAKARA, and Thermo Scientific). Some reprinted materials are quoted and some instruction manuals, with necessary modification, are cited. The related sources are indicated, and the references are listed at the end of this book. Manuscript proofreading was in part provided by Prof. Ting Zhu before printing. We thank him for his devotion and hard work.

Finally, we would like to say that despite all our efforts to complete this textbook, it is inevitable that some inappropriate expressions or errors are included. Therefore, any suggestions from readers will definitely be welcome so that we can make these improvements in the next edition.

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Experiment 1

Preparation of Plasmid DNA

1 Objectives

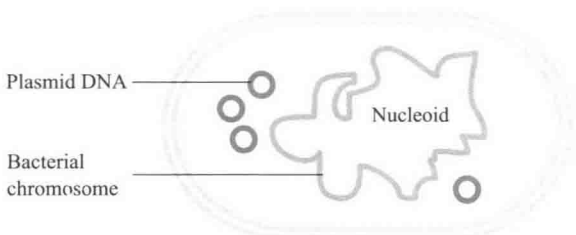
- Learn the characteristics of plasmid DNA
- Understand the principles of purification and quantification of plasmid DNA
- Learn the method of plasmid DNA minipreparation by alkaline lysis and the measurement of DNA concentration by spectrophotometer

2 Background and Concepts

2.1 Basic features of plasmids

Plasmids are small double-stranded circular DNA molecules that are extrachromosomal and capable of replicating independently of chromosomes (Fig. 1.1). They are largely found in bacteria and sometimes occur in eukaryotic organisms. Some plasmids can be transferred from one organism to another, and some are capable of integrating into the host genome. Plasmid sizes range from 1 to over 300 kb. Some small plasmids and host cell genomic DNA share a set of replicative enzymes. Some of the large plasmids carry genes encoding enzymes that are specific for plasmid replication.

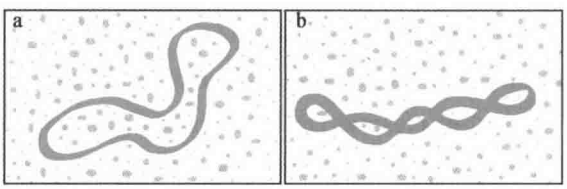
Plasmids are commonly used as vectors for cloning genes. A number of artificially modified plasmids are employed as cloning vectors.



● **Fig. 1.1 Illustration of a bacterium containing plasmid DNA.** Plasmids: small, circular DNA molecules within the host bacterium.

2.2 Plasmid topology

Plasmids can be divided into several types depending on their configuration. If both strands of plasmid DNA are intact circles, the molecules are described as **covalently closed circle DNA (cccDNA)**. If one nucleotide strand is broken, the resulting plasmids are described as **open circle DNA (ocDNA)**. When isolated from cells, cccDNAs often have two configurations, i.e., relaxed and supercoiled (Fig. 1.2 shows the structure). Supercoiled plasmids make up the majority of prepared plasmids.



● **Fig. 1.2 Cartoons showing the topology of a plasmid.** a. Relaxed circle plasmid DNA. b. Super-coiled plasmid DNA. Both of these molecules are from common bacteria. *Schlick T., (1995)* Current Opinion in Structural Biology *5:245-262. With permission from Elsevier.*

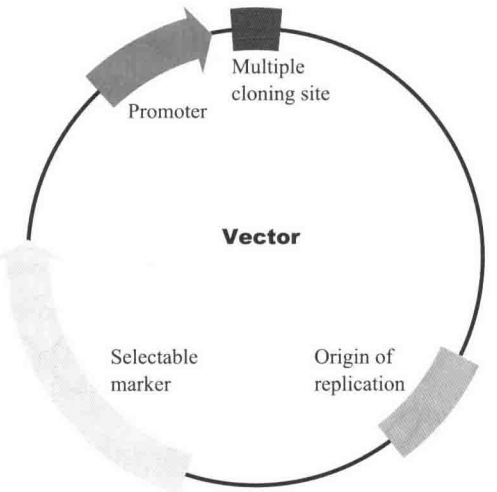
2.3 Plasmid types

One way to classify plasmids is by the copy number in host cells. A **stringent plasmid** occurs at a low copy number in cells. Stringent plasmids only replicate along with the bacterial chromosome and are present as single copies or at most several copies per cell. A **relaxed plasmid** is one that replicates independently of the bacterial chromosome. Relaxed plasmids are present at a high copy number (approximately 10 to 500 copies per cell). Multicopy plasmids have the property of being able to replicate continuously, even when protein synthesis in the host cell is restrained by antibiotics.

2.4 Vectors

Bacteriophages, plasmids, and other agents serve as tools for transferring a foreign or modified gene to a living organism. These molecules that are used as carriers in gene cloning experiments are termed **vectors**. The vector is generally a DNA molecule that serves as the "backbone". Vectors are typically used to isolate, multiply, or express the insert (foreign gene) in target cells. Therefore, a vector usually possesses elements for DNA manipulation and autonomous replication in host cells. Identifying and screening the transformed host cells also depend on the properties

of the vector and sometimes the insert. Commonly used vectors contain a selectable marker (such as an antibiotic resistance gene), a multiple cloning site (MCS) or poly-linker, and the origin of replication (*ori*). Some vectors also contain additional genes for selection, such as the *lacZ'* gene (Fig. A.3, see App. Ⅱ). Fig. 1.3 shows the typical structure of a vector.



● **Fig. 1.3 The structure of a vector.**

2.5 Methods for bacterial lysis

An obvious prerequisite for plasmid DNA purification is cell disruption. The most commonly used methods are as follows:

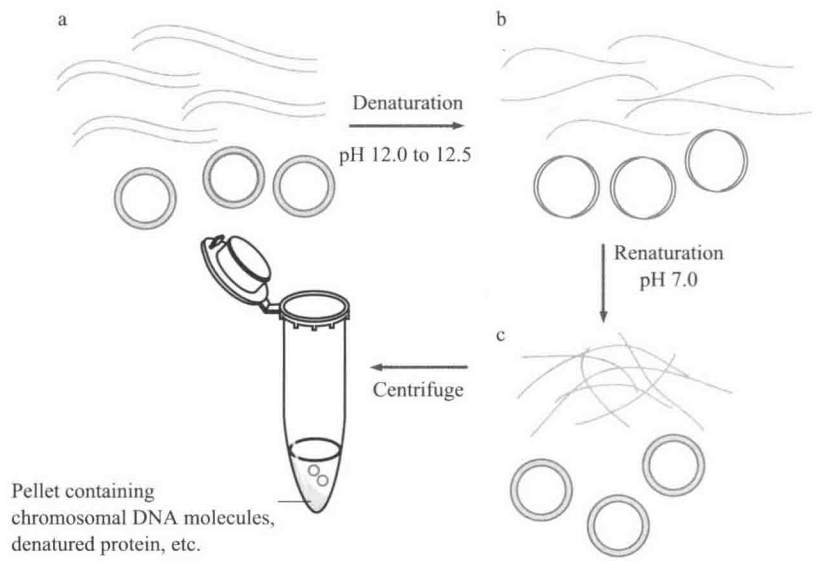
1. Alkaline lysis: 0.2 M NaOH+1% SDS (*m*/*V*) is used.
2. Boiling lysis: bacterial cells are boiled for 40 s.
3. Lysis with SDS:10% SDS is generally used for large-scale plasmid purification.

2.6 Principle of plasmid DNA purification

Based on the properties of DNA molecules, heat, organic solutions (such as urea), and pH extremes can denature double-stranded DNA. After the temperature or pH is returned to the normal range, the two strands rewind, or anneal, to form the intact duplex. Therefore, chromosomal DNA in cell extracts remains in a high-molecular-weight form but can be denatured over a narrow pH range (12.0-12.5). In contrast, cccDNAs are partially denatured. Upon neutralization with acidic potassium acetate, the chromosomal DNA renatures with difficulty and aggregates to form an insoluble network along with precipitated denatured proteins, but ccc plasmid DNA remains in

a native state after rapid renaturation to its supercoiled form (Fig. 1.4). Cleared lysate containing plasmid DNA can then be obtained by centrifugation. Plasmids are concentrated by ethanol precipitation or recovered using a commercial column with a suitable solid-phase support for plasmid adsorption and elution. RNA can be destroyed using ribonuclease in conjunction with the alkaline lysis method.

Many factors affect the yield and purity of plasmid DNA. Both dissolution of the cells and incomplete lysis result in greatly reduced recoveries of plasmid DNA. Appropriate operation and careful control of the alkaline denaturation and renaturation steps can prevent contamination.



● **Fig. 1.4 Principle of plasmid purification using the alkaline method.** a. Chromosomal DNA as linear fragments; plasmid as covalently closed circular DNA. b. Denatured plasmid DNA strands remain interlocked; linear DNA fragments separate. c. If acid is added, interlocked plasmid strands snap together; bacterial genomic DNA strands aggregate into a tangled mass with proteins, membranes, and other molecules.

2.7 Determination of DNA concentration

Several methods are used to determine the concentration of DNA in solution. One method is measuring UV absorbance (optical density) by spectrophotometer. Another method is comparing the fluorescence intensity of the DNA bands of unknown concentration with that of a standard DNA marker in agarose gels stained with ethidium bromide (EtBr) after electrophoresis. Finally, DNA concentration can be calculated by determining the fluorescence intensity of intercalating fluorescent dyes (such as Picogreen™, Molecular Probes) in the DNA samples.

UV absorbance spectrophotometry

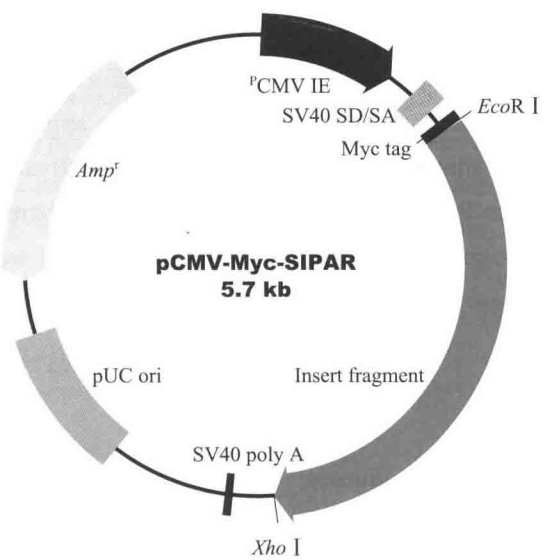
This method is based on the strong absorbance of DNA nitrogenous bases (A, T, G, C) at a wavelength of 260 nm. The absorption coefficient varies according to different pH levels of a solution. Given the effect of solvents, a neutral pH solution is not only normally used in sample purification but is also used in dilutions for the measurement of DNA concentration.

At a wavelength of 260 nm, an absorbance of 1.0 corresponds to 50 μg of double-stranded DNA per mL. Because the absorption maxima for DNA and protein are 260 nm and 280 nm, respectively, the absorbance value is also measured at 280 nm to determine the purity of a DNA sample. With a pure sample of DNA, the ratio of the absorbance at 260 nm and 280 nm (A_{260}/A_{280}) is 1.8. Ratios less than 1.8 indicate that the preparation may be contaminated with protein or other materials. Otherwise, the contaminate may be RNA or other substances, e.g., the buffer components used for lysis, that remain in the DNA eluate.

3 Materials and Equipment

3.1 Materials

E. coli DH5α harboring pCMV-Myc-SIPAR (Fig. 1.5) is used in this experiment.



● **Fig. 1.5 Restriction map of pCMV-Myc-SIPAR.**

■ pCMV-Myc(Clontech, TaKaRa) is a type of expression vector for mammalian cells

- Suitable host strains: DH5α, HB101, and other general-purpose strains
- Selectable marker: the plasmid confers its *E. coli* hosts with resistance to ampicillin (100 mg/mL)
- *E. coli* replication origin: pUC
- Copy number: ~500

3.2 Reagents

LB (Lygogeny broth or Luria-Bertani) medium, culture medium for *E. coli*

Tryptone	10 g/L
Yeast extract	5 g/L
NaCl	10 g/L

Adjust pH to 7.0 with sodium hydroxide, autoclave, and store at 4℃ after cooling. Add ampicillin to a final concentration of 50 μg/mL before use. For preparing LB agar plates, add 15 g of agar to 1 L of medium before autoclaving. Pour the medium into sterilized plates after the agar flask is cool enough to hold. Add antibiotic before pouring plates if necessary.

Plasmid DNA purification

Any commercially available kits for plasmid DNA purification can be used. In this experiment, the TIANprep Mini Plasmid Kit (TIANGEN) is used. This kit is designed to extract and purify plasmid DNA from *E. coli* cells. The cells are lysed using the alkaline/SDS procedure. The lysate is then applied to a centrifugal column with specific silicon adsorption material that selectively binds plasmid DNA in high-salt buffer. Proteins and other contaminants are removed with Wash Buffers. In the following step, purified DNA is easily eluted with Elution Buffer or deionized water and is suitable for all routine downstream applications.

Buffer BL

Buffer P1

50 mM	glucose
10 mM	EDTA
25 mM	Tris-HCl, pH 8.0

Buffer P2

0.2 M	NaOH
1%	SDS (*m/V*)

Buffer P3

1 M	KOAc
4 M	guanidine hydrochloride, pH 5.3

Wash buffer

1 mM	Tris base
80%	ethanol

Elution buffer

10 mM	Tris-HCl
1 mM	EDTA, pH 8.0

Notes

Add RNase to Buffer P1, mix, and store at 2-8℃.

Add a 4-fold volume of ethanol to the Wash buffer before use!

3.3 Equipment

Constant-temperature shaker, microcentrifuge, pipette and vortexer

4 Procedures

4.1 Beforehand

1. Pick a single, well-isolated colony from a fresh agar plate (containing ampicillin at 50 μg/mL) and place the colony into 3-5 mL of LB medium (containing ampicillin at 50 μg/mL). Incubate the culture overnight at 37℃ with vigorous shaking.
2. Inoculate 200 mL of LB medium (containing ampicillin, 50 μg/mL) with 1-2 mL of the fresh overnight culture containing *E. coli* DH5α harboring the desired plasmid, pCMV-Myc-SIPAR. Incubate the culture at 37℃ overnight in a shaking incubator (200 rpm).

4.2 Purification of plasmid DNA

Notes

Carefully use pipettes in each procedure.

To prevent shearing of DNA, do not vigorously vortex after adding Buffer P2 to the tube. Otherwise, both plasmid DNA and genomic DNA will be broken. Mix the solution only by inverting the tubes.

Use a fresh tip for each reagent. However, it is unnecessary to change tips when adding the same reagent. The same tip may be used for all tubes, provided that the tip has not been contaminated by any solution already in the tubes.

1. Column equilibration: add 500 μL of **Buffer BL** to the mini-spin column. Centrifuge for 1 min at 12,000 rpm. Discard the flow-through, and place the column into the same collection tube.
2. Shake the culture to resuspend the *E. coli* cells.
3. Label a 1.5-mL microcentrifuge tube with your group number or initials. Transfer 1.4 mL of the *E. coli*/pCMV-Myc-SIPAR overnight suspension into the tube.
4. Harvest the cells by balanced centrifugation for 30 s at 12,000 rpm. Discard the supernatant from the tube into a waste beaker. *Take care not to disturb the cell pellets.* Invert the tubes and blot onto a clean paper towel to remove as much of the remaining supernatant as possible.
5. Repeat the steps above to collect more *E. coli* cells. Discard the supernatant after centrifugation.
6. Add 250 μL of ice-cold **Buffer P1** to the tube. Resuspend the pellets by vortexing or pipetting up and down several times. Hold the tubes up to the light to check that the suspension is homogeneous and that no visible clumps of cells remain.
   Without sufficient suspension, the efficiency of the plasmid purification will be poor!
7. Add 250 μL of room temperature **Buffer P2** to the tube. Close the cap, immediately invert the tube gently several times, and incubate the tube on ice for 1-2 min. *Do not exceed this period!* The solution will become relatively clear with bacterial lysis.
8. Add 350 μL of **Buffer P3** to the tube. Close the cap and invert the tube gently several times. A white precipitate will immediately appear. Place the tube on ice (or at room temperature) for 5 min and centrifuge at 12,000 rpm for 10 min. Befor centrifugation, *incubate the tube on ice for better results!*
9. Transfer the supernatant from step 8 to a mini-spin column. Centrifuge at 12,000 rpm for 30 s. Discard waste liquid in the collection tube and return the column to the collection tube.
10. Wash the column by adding 700 μL of **Wash buffer** to the column and centrifuge at 12,000 rpm for 1 min. Discard the flow-through and return the column to the collection tube.
    Add ethanol to Wash buffer before use! Without ethanol, plasmid DNA will be washed down with other contaminants. The recovery efficiency will be poor!
11. Wash the column by adding 500 μL of **Wash buffer** and centrifuge at 12,000 rpm for 1 min. Discard the flow-through and return the column to the collec-

tion tube. Centrifuge again at 12,000 rpm for 2 min. Eliminate the wash buffer as thoroughly as possible. *Prolong the centrifugation time or use a pipette to remove the wash solution if necessary. Open the cap of the collection tube and keep it at room temperature for 1-2 min. The ethanol present in the wash buffer will impact the subsequent enzyme-catalyzed reactions.*

12. Carefully transfer the column to a new 1.5-mL microcentrifuge tube. Add 50 μL of **Elution buffer or water** into the column, allow it to stand at room temperature for 5 min, and centrifuge at 12,000 rpm for 1 min. *Elution buffer or water should be added in the middle of the adsorption material to guarantee that all the plasmid DNA is recovered. To increase the recovery efficiency, increase the elution volume or elution times if necessary. RNase A (0.5 μL, 10 mg/mL) can be added to the purified plasmid to completely eliminate RNA. If the molecular weight of the purified plasmid exceeds 10 kb, warm the Elution buffer or water to 70°C prior to elution.*

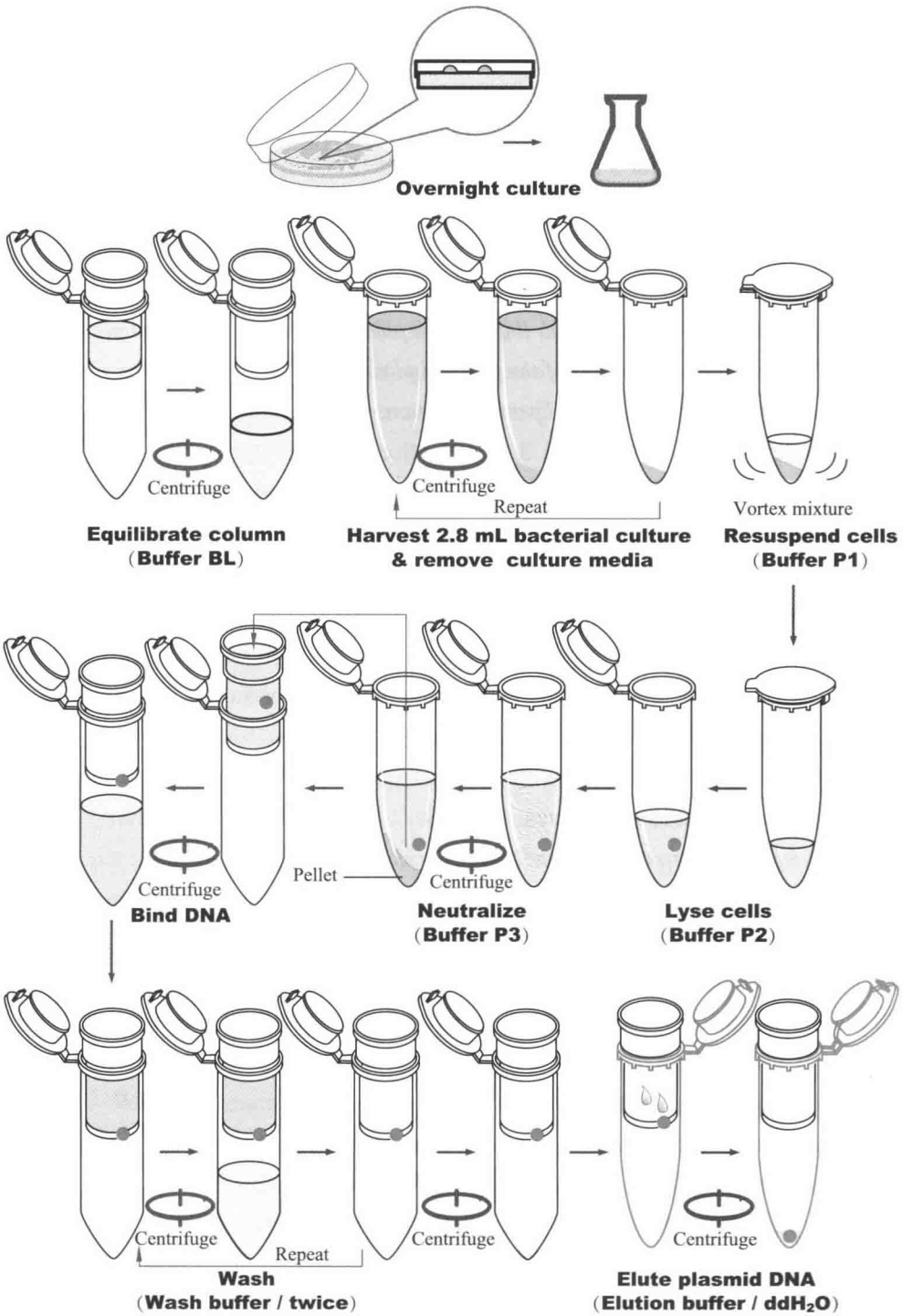
4.3 Determination of the DNA concentration using a UV spectrophotometer

1. Add 4 μL of plasmid to 96 μL of ddH_2O (double distilled water) (1:25 dilution) in a 1.5-mL microcentrifuge tube.
2. Measure a blank with only ddH_2O in a cuvette and set the A_{260} and A_{280} values of the UV spectrophotometer to zero.
3. Measure sample: remove ddH_2O and add the sample to the cuvette, then record the absorbance at 260 nm and 280 nm.
4. A_{260} is used to calculate the nucleic acid concentration of samples:
   if $A_{260}=1$,
   the concentration of double-stranded DNA is 50 μg/mL, and
   the concentration of single-stranded DNA is 33 μg/mL.
5. The ratio of A_{260}/A_{280} can be used to estimate the purity of nucleic acid samples. Generally, the ratio of pure DNA is 1.8. If the ratio is less than 1.8, DNA may be contaminated by protein or other material.
6. Record the A_{260} and A_{280} values and calculate the concentration and purity of the plasmid DNA according to the following formula:
   dsDNA=50 × (OD_{260}) × dilution factor (unit: μg/mL).

UV spectrophotometer

EPPENDORF BioPhotometer The concentration shown in the spectrophotometer

has already taken the dilution factor into account (See App. Ⅲ.4). NanoDrop (Thermo Scientific) or NanoPhotometer (IMPLEN) spectrophotometers can also be used to determine DNA concentration using a similar procedure but without requiring dilution.



● Fig. 1.6 Flow diagram of plasmid DNA purification.